Lung Cancer xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Lung Cancer

journal homepage: www.elsevier.com/locate/lungcan

Silencing of miR-137 by aberrant promoter hypermethylation in surgically resected lung cancer

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ARTICLE INFO

Article history:
Received 18 February 2015
Received in revised form 13 April 2015
Accepted 13 May 2015

Keywords:
Promoter methylation
microRNA
Tumor suppressor
Silencing
MIR137
miR-137
Lung cancer

ABSTRACT

Background: Recent studies demonstrated that miR-137 is downregulated in various tumors, and that it functions as a tumor suppressor. miR-137 could be silenced by its aberrant promoter hypermethylation. The purpose of this study was to investigate the significance of *MIR137* promoter methylation on its expression in lung cancer.
Methods: Lung cancer cell lines were treated with either a DNA methyltransferase inhibitor (5-azacytidine, AZA) and/or an HDAC inhibitor (trichostatin A, TSA) to determine whether miR-137 expression was reactivated. Paired lung tumor and adjacent non-tumor lung tissues were obtained ($n = 50$). Quantitative methylation-specific PCR and bisulfite sequencing were used to analyze the methylation status of *MIR137*, and real-time RT-PCR was performed to analyze miR-137 expression.
Results: miR-137 was reactivated by treatment with either AZA and/or TSA in lung cancer cell lines. Methylation-specific PCR showed increased *MIR137* promoter methylation in lung tumors compared with adjacent non-tumor tissues, which was further validated by bisulfite sequencing. The expression of miR-137 was downregulated significantly in lung tumors, which was correlated with level of *MIR137* promoter methylation inversely.
Conclusions: miR-137 downregulation was related to its promoter hypermethylation in lung cancer. Further studies are needed to assess its value as a prognostic factor and potential therapeutic applications in lung cancer.

1. Introduction

Lung cancer remains the leading cause of cancer-related mortality worldwide, and accounts for 27% of all cancer-related deaths in the United States [1]. In patients with early stage non-small-cell lung cancer (NSCLC), surgical resection is the curative treatment of choice.miR-137 is located on chromosome 1p22, and is downregulated frequently in several types of cancers including colorectal cancer, gastric cancer, glioblastoma, and NSCLC [2–6]. miR-137 has a tumor suppressor function by targeting several oncogenic mRNA to inhibit proliferation and invasion by inducing apoptosis and cell cycle arrest in cancer cells [3,7]. Previous studies suggested that miR-137 silencing might be associated with promoter hypermethylation of the *MIR137* gene [2,3,8]. Furthermore, hypermethylation of the *MIR137* promoter region was associated with poor prognosis in clinical practice [9,10]. However, little is known how the silencing of miR-137 expression relates to its promoter methylation in lung cancer.

Therefore, in the present study we investigated whether miR-137 silencing is associated with the promoter hypermethylation of *MIR137* in lung cancer. Moreover, *MIR137* promoter methylation

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http://dx.doi.org/10.1016/j.lungcan.2015.05.011
0169-5002/

and miR-137 expression were compared in paired lung tumor and adjacent non-tumor tissue from patients with surgically resected primary lung cancer.

2. Materials and methods

2.1. Cell lines and tissues

Human NSCLC cell lines (A549, H292, H226, and H460) were obtained from the American Type Culture Collection (Manassas, VA, USA), and paired primary NSCLC tumor and adjacent non-tumor tissues were obtained from the Seoul St. Mary's Hospital Biobank (Seoul, Korea). The patients provided informed consent and underwent curative surgical resection at Seoul St. Mary's Hospital between February 2009 and December 2013. The tissues were snap-frozen and stored at −80 °C until use. Patients' clinicopathological characteristics were reviewed from medical records, including demographic data, histological type, and pathological (TNM) stage. The Institutional Review Board of Seoul St. Mary's Hospital, The Catholic University of Korea (approval no. KC11SISI0613) approved the study protocol.

2.2. Genomic DNA and RNA extraction from cell lines and tissues

Cell lines were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified water-jacketed incubator containing 5% CO_2.

A surgical pathologist (Lee KY) evaluated the paired tumor and adjacent non-tumor tissues histologically after tissue specimens had been stained with hematoxylin and eosin (H&E). All specimens including tumor and adjacent non-tumor tissues were macro-dissected by the surgical pathologist to yield tissue containing the maximum number of tumor cells in lung tumor specimens, and sections from adjacent non-tumor tissues were also examined to ensure an appropriate fraction of epithelial cells, and absence of cancer or dysplastic cells.

DNA and RNA were extracted from tissues and cell lines using the DNeasy Blood and Tissue Kit (Qiagen, Austin, TX, USA) and TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA), respectively, according to the manufacturer's instructions.

2.3. Treatment with a DNA methyltransferase inhibitor and an HDAC inhibitor

Cell lines were plated at a low density (9×10^4 cells/well in six-well plates) 24 h before treatment, and were then incubated with either 5 μM 5-azacytidine (AZA; DNA methyltransferase inhibitor; Sigma, St. Louis, MO, USA) and/or 500 nM trichostatin A (TSA; HDAC inhibitor; Sigma, St. Louis, MO, USA) for 24 h. These chemicals are used commonly to determine whether DNA methylation or histone modifications influence gene expression. The same volume of DMSO was used to treat cells as a negative control.

2.4. Analysis of MIR137 promoter lesion and sodium bisulfite conversion

MIR137 primers for methylation-specific PCR and bisulfite sequencing were designed after the location and structure of the *MIR137* CpG island were identified using Methyl Primer Express® Software Version 1.0 (Applied Biosystems, Foster City, CA, USA) and MethPrimer (www.urogene.org/methprimer). DNA extracted from cell lines and tissues was treated with sodium bisulfite (EpiTect Bisulfite Kit, Qiagen) for methylation-specific PCR and bisulfite sequencing.

2.5. Methylation-specific PCR

Real-time PCR was used to analyze *MIR137* methylation in bisulfite-modified DNA from lung tumor and adjacent non-tumor tissues. The following primer pairs were used: forward, GTA GCG GTA GCG GTA GTA GC; and reverse, ACC GCT AAT ACT CTC CTC GA. The methylation level was normalized with *β-actin* gene. A region of *β-actin* devoid of any CpG dinucleotide was amplified using the following primers: forward, TGG TGA TGG AGG AGG TTT AGT AAG T; and reverse, AAC CAA TAA AAC CTA CTC CTC CCT TAA. The ratios between the methylation levels of *MIR137* and *β-actin* were used as a measure for representing the relative level of methylation of promoter DNA (*MIR137*/*β-actin*). Bisulfite-modified DNA was PCR-amplified using the following reaction conditions: GoTaq® qPCR Master Mix (Promega, Madison, WI), 10 nM of each primer, and 250 ng of template DNA in a final volume of 20 μl. PCR was performed using Exicycler™ 96 (Bioneer, Daejeon, Korea) with an initial denaturation step of 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s.

2.6. Bisulfite sequencing of the promoter region of MIR137

Bisulfite-modified DNA at the *MIR137* promoter region was amplified using the following primers: forward, AAG GTT TTG AGT AGT TTG GGA G; and reverse, CCA ACC TCT TTC AAA AAA ATT C. The PCR product was then sequenced using an Applied Biosystems® 3730xl DNA Analyzer.

2.7. Real-time RT-PCR to analyze miR-137 expression

One-hundred nanograms of RNA extracted from cell lines or tissues were used to synthesize cDNA using a TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems). miR-137 expression was then quantified using TaqMan® MicroRNA Assays (Applied Biosystems) on a ViiA™ 7 Real-Time PCR System (Applied Biosystems) according to the manufacturer's instructions. The expression of miR-137 was normalized to that of U6.

2.8. Statistical analysis

Summary statistics are presented as percentages for categorical variables, and as means and standard deviations for continuous variables. Differences between the tumor and adjacent non-tumor groups from the same individuals were evaluated using Wilcoxon signed-rank tests. Univariate Pearson's correlation coefficients were calculated to analyze correlations. The prognostic significance of factors affecting overall survival was determined using the Cox proportional hazard ratio model, whereas factors affecting the cumulative incidence of recurrence were determined using the semi-parametric proportional hazards model for sub-distributed competing risk analysis (SAS macro '%PSHREG'). All reported *P* values are two-tailed, with a *P* value of 0.05 indicating statistical significance. Statistical analyses were performed using SAS, version 9.3 for Windows (SAS Institute, Cary, NC, USA).

3. Results

3.1. Patients' characteristics

The characteristics of the 50 surgically resected NSCLC (32 adenocarcinomas, 14 squamous cell carcinomas, two poorly differentiated carcinomas, one adenosquamous cell carcinoma, and one double primary adenocarcinoma and squamous cell carcinoma) patients are shown in Table 1. The mean age was 65.3 years, and there were 30 (60%) males and 20 (40%) females. The proportion

Please cite this article in press as: Kang N, et al. Silencing of miR-137 by aberrant promoter hypermethylation in surgically resected lung cancer. Lung Cancer (2015), http://dx.doi.org/10.1016/j.lungcan.2015.05.011